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The effect of light on the level of acetylcholine in seedlings of the wild-type and phytochrome mutants of tomato (*Lycopersicon esculentum* Mill.)

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Key words: acetylcholine, gas chromatography, pyrolysis, phytochrome, photomorphogenic mutants, tomato, *Lycopersicon esculentum*

Abstract

Applying the method of pyrolysis coupled with gas chromatography (PYR-GC) the content of endogenous acetylcholine (ACh) was investigated in the extracts obtained from tomato (*Lycopersicon esculentum* Mill.). Seven-day-old seedlings of wild type (WT) and phytochrome mutants *au* (*aurea*), *hp* (*high pigment*), *fri* (*far-red light insensitive*) and *tri* (*temporarily red light insensitive*) were studied. In the analyzed material the presence of choline and acetylcholine was discovered. The highest content of ACh (381 mmole/g of fresh weight) was found in tomato cotyledons, whereas the lowest amount (162 nmole/g of fresh weight) in roots. The level of ACh in the plants grown under the continuous light was higher than in etiolated ones. However, no considerable differences in the concentrations of ACh in *au* and *tri* seedlings grown under the continuous light and in darkness were observed. The irradiation of etiolated seedlings of wild type with red light was accompanied by the increase of endogenous level of ACh. The pulse of far-red light applied directly after red light reversed this stimulating effect. A similar effect of both light wavelengths on the content of ACh was also found in the case of the *tri* mutant. On the other hand, in the case of *fri* mutant, pulse of red light caused the drop in the content of ACh, whereas far-red applied after red light caused visible increase in the level of the investigated substance. In tissues of *au* mutant no effect of red and far-red lights on the concentration of ACh was established.

List of abbreviations: ACh - acetylcholine; AChE - acetylcholinesterase; AChR - acetylcholine receptor; ChAT - choline acetyltransferase; FR - far red light; PhyA - labile phytochrome; PhyB - stable phytochrome; *PHYA* - gene of the labile phytochrome, *PHYB* - gene of the stable phytochrome; R - red light

Introduction

The cholinergic system was originally discovered in animals. It plays a basic function in the processes of transmitting information received by receptors along the neurones in the form of electric impulses. This system is composed of four elements: acetylcholine (ACh) and its receptors (AChR), choline acetyltransferase (ChAT) and acetylcholinesterase (AChE), enzymes, which take part in synthesis and hydrolysis of ACh, respectively (Hartmann and Gupta 1989).

The presence of ACh was found in the tissues of more than 50 plant species belonging to all the major systematic groups (Miura *et al.* 1982, and for review see Tretyn and Kendrick 1991). It has been postulated that the mechanism of action of plant cholinergic system is controlled by phytochrome

(Jaffe 1970, 1976, Hartmann and Gupta 1989; Tretyn and Kendrick 1991) - the photoreceptor regulating the growth and development of plants (Kendrick and Kronenberg 1994, Furuya and Schäfer 1996; Quail 1997). It was found that ACh can mimic the action of red light in the regulation of some photomorphogenetic phenomena in plants (Jaffe 1970, 1972, Hartmann and Gupta 1989, Tretyn and Kendrick 1991). So, it seems that ACh may be a specific secondary transmitter of a phytochrome action or an element of light-controlled signal transduction pathway (Jaffe 1970).

In the case of several species of lower and higher plants the structure of genes encoding the proteinaceous component of phytochrome were recognized (Quail 1994a,b, Pratt 1995). The best known plants are *Arabidopsis thaliana*, *Avena sativa* (oat) and *Lycopersicon esculentum* (tomato). In *A. thaliana* there are five genes encoding the proteinaceous component of separate types of phytochrome denoted as PhyA, B, C, D and E (Mathews and Sharrock 1997, Quail *et al.* 1997). Also, in the tomato genome at least five phytochrome genes which encode PhyA, B1, B2, E and F were identified (Pratt 1995, Pratt *et al.* 1997). It was established that genes *PHYA* and *PHYE* of tomato have their equivalents in *Arabidopsis*. Also, two other tomato genes - *PHYB1* and *PHYB2* are similar to the gene *PHYB* in *Arabidopsis* (Pratt 1995, Pratt *et al.* 1997). Apart from *PHYF*, in tomato there may occur from four to eight additional, so far unidentified genes, which have no equivalents in other plant species. Both, in *Arabidopsis* as well as in tomato, all the phytochrome genes may encode two physiologically separate forms of this photoreceptor - a labile and a stable one (Furuya 1993, Quail 1994a,b). In both cases the labile phytochrome which is synthesized in darkness (denoted as PhyA) is rapidly degraded on light. On the other hand, the content of a stable phytochrome (encoded by the remaining type of the above mentioned genes), occurring in green plants is relatively constant independently on light conditions (Furuya 1993, Quail 1994a,b, 1997).

Many mutations influencing the functioning of a labile as well as a stable form of phytochrome were identified in tomato (van Tuinen *et al.* 1995, 1996, Kerckhoffs 1996, Kendrick *et al.* 1997). The mutants of the light-induced chain of the signal transduction are also known (Kendrick *et al.* 1994, 1997). Applying the above mentioned types of mutants for the investigations allowed us to better understanding the role of phytochrome in the process of ACh synthesis in plants. Due to this we have shown, for the first time, that both forms (labile and stable) of phytochrome take part in the control of endogenous level of ACh in plant tissues.

Material and Methods

Plant material

All studies were performed on 7-day-old etiolated or green tomato (*Lycopersicon esculentum* Mill.) seedlings of the wild type (WT), as well as *au* (*aurea*) and *hp* (*high pigment*) mutants, all *cv.* Ailsa Craig (AC). Furthermore, two other tomato mutants: *fri* (*far-red light insensitive*) *cv.* Money Maker (MM) and *tri* (*temporarily red light insensitive*) *cv.* GT (tobacco mosaic virus resistant tomato breeding line of MM) were used.

Tomato seeds were sterilized for 2 min with 2 % solution of sodium perchlorate. After intensive washing the seeds were soaked for 2 h in sterilized distilled water. Then they were sown into plastic containers with 0.8 % agar covered with a thin film of steriliezed, 10 times diluted Murashige and Skoog (1962) nutrient solution supplemented with 10 mM potassium nitrate (Peters, 1992). Cultivation of seedlings was performed in growth chambers at 26 °C, in darkness or under continuous white light. A fraction of the dark-grown seedlings was treated with red (R) and far red (FR) light. All experiments performed on etiolated seedlings were conducted under safe green light.

Light sources

For continuous white light irradiation cool-white fluorescent tubes (fluence rate 18.3 $W \cdot m^{-2}$) were used. A specially constructed projector with a revolving holder containing interference filters was used for monochromatic irradiation. The light source was a 250 W quires-lamp with the optical system from a Diaprex projector. The fluence rates used were 1.08 and 0.87 $W \cdot m^{-2}$ for R ($_{max.}$= 660 nm) and FR ($_{max.}$= 730 nm) light, respectively.

Isolation and purification of Ach

Isolation and purification of ACh was performed according to Hanin and Jenden (1969) with modification introduced for plant material by Tretyn *et al.* (1997). Tomato seedlings (500 mg) were immediately immersed in liquid nitrogen and pulverized using a mortar and pestle. The pulverized material was subsequently homogenized with ice-cold extraction mixture containing 15 % 1 N formic acid and 85 % acetone (5 ml per 500 mg of tissue). Three 15 s bursts from a homogenizer were used with 15 s cooling in an ice bath between bursts. The homogenate was chilled for 30 min in an ice bath, after which it was centrifuged for 30 min at 15,000 g. Homogenate was filtered, and filtrate was transferred to new tubes and washed with an equal volume of diethyl ether. After brief centrifugation, the ether phase was discarded. The washing was repeated twice, and the ether remaining after centrifugation was removed with a stream of nitrogen. To each 1 ml of an aqueous phase of the homogenate, 0.1 ml of 1 mM tetraethyl-ammonium chloride and 1 ml of cold 2 % ammonium reineckate were added. The contents of the tubes were agitated, chilled for 40 min in an ice bath, and then centrifuged for 20 min at 0 °C. The precipitate was vacuum dried and dissolved in 1 ml of 5 mM silver p-toluene-sulphonate in acetonitrile, and after agitation for 1 min centrifuged. The supernatant was transferred to Eppendorf tubes and after being dried with nitrogen, subjected to pyrolysis.

Pyrolysis

Purified and dried plant extracts were dissolved in 50 µl of acetonitrile. From the obtained solution 4 µl samples were taken for pyrolysis. It was conducted at 500 °C for 30 s using PYR-2A type Shimadzu (Japan) pyrolyser.

Gas chromatography

Gas chromatography of pyrolysed plant extracts was performed on the capillary column (25 m x 0.32 mm id.) coated by Permabound CW20M-DF-0.25 (Marcherey-Nagel, Germany) using Shimadzu GC-14A chromatograph (Shimadzu, Japan). The chromatograph was equipped with a flame ionization detector (FID). Analyses were conducted under the following conditions: column temperature, 50 °C for 2 min, elevated to 160 °C at 20 °C/min, and then maintained at 160 °C. Injector and detector temperatures were both 170 °C. The following gas flows were used: N_2 - 150 kPa (carrier gas) and 100 kPa (make up), H_2 - 50 kPa; air 50 kPa and split 60 ml/min. The retention times and the area of peaks were recorded automatically using a Shimadzu C-R6A Chromatopac integrator (Shimadzu, Japan) coupled with the chromatograph.

Each experiment was repeated three times, with at least 3 measurements in each experiment. Means and standard errors were calculated.

Results

Up to now only several authors have used gas chromatography for the analysis of ACh in plant material (Hartmann and Kilbinger 1974a, Miura and Shih 1984, Tretyn *et al.* 1987, 1997, Tretyn A. and Tretyn M. 1990, Momonoki Y.S. and Momonoki T. 1991, 1992). Because of the non-volatile properties of this substance (similarly as in the case of other quaternary amines) it should be transformed into tertiary amine before commencing the chromatographic determination. This consists of the detachment of one methyl group (CH_3-), which results in the formation of volatile 2-methylaminoethyl acetate. This effect may be obtained by the way of a chemical degradation of ACh or as a result of its thermal decomposition - pyrolysis. It follows from our previous investigations that far better effects are obtained applying the second of the above mentioned methods (Tretyn *et al.* 1987, 1997). Pyrolysis-gas chromatography (PYR-GC) is characterized by high sensitivity to choline and its derivatives and great repeatability of results (Momonoki Y.S. and Momonoki T. 1991, Tretyn *et al.* 1997).

In animal tissues apart from choline (Ch) there may occur several of its esters such as acetylcholine (ACh), propionylcholine (PCh) and butyrylcholine (BCh). As it was shown in the preliminary investigations carried out according the appropriate standards these all substances could be identified and determined quantitatively using the pyrolysis coupled with gas chromatography (PYR-GC). Comparing the retention times of pyrolysis products of

Ch, ACh, PCh, and BCh it was established that they do not overlap which makes possible their simultaneous determination (Fig. 1A). However, in the extracts from tomato seedlings only the presence of Ch and ACh (Fig. 1B) was established.

The highest concentration of ACh (381 nmole/g of fresh weight) was found in cotyledons of seven-day-old etiolated tomato seedlings (Fig. 2). It was about 82 % higher as compared with the whole seedlings. On the other hand, the content of ACh in

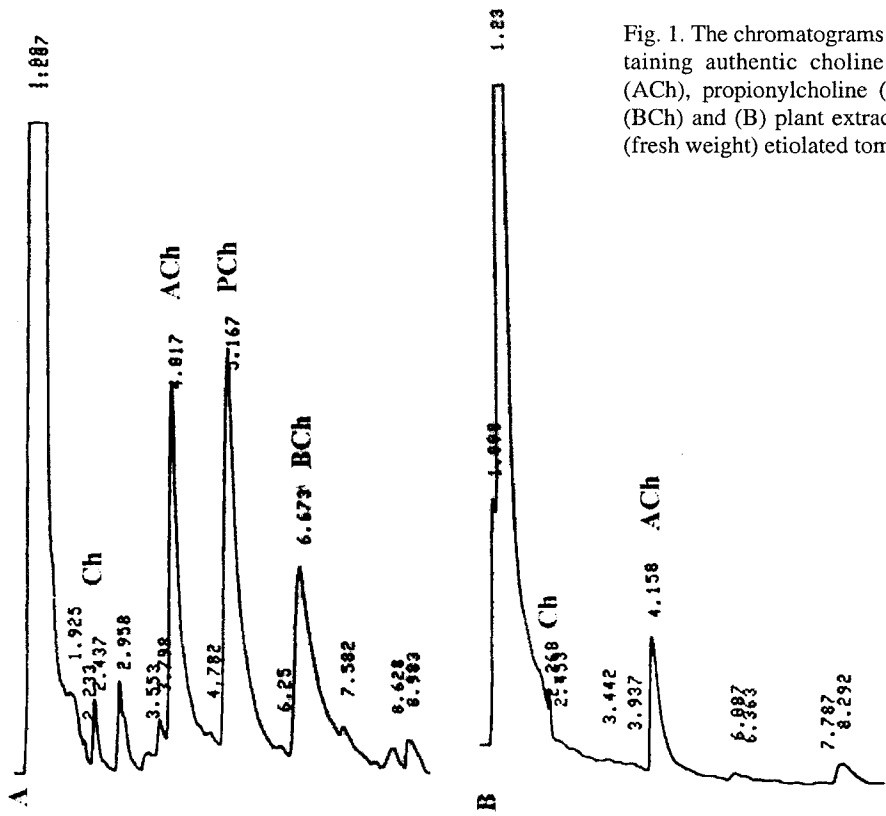
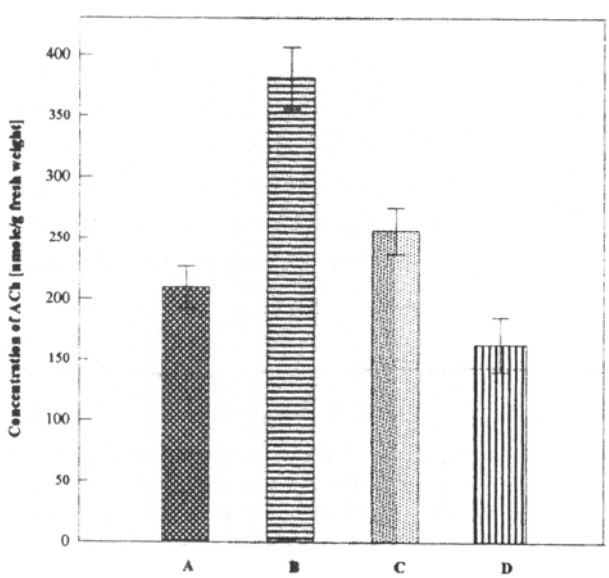


Fig. 1. The chromatograms (A) of a mixture containing authentic choline (Ch), acetylcholine (ACh), propionylcholine (PCh), butyrylcholine (BCh) and (B) plant extract isolated from 0,5 g (fresh weight) etiolated tomato seedlings.



roots was about 23 % lower as compared with whole seedlings (Fig. 2). Similar differences in the level of the investigated substance were observed in the organs of seedlings grown on continuous light (data not shown).

The concentration of ACh in the tissues of tomato seedlings of wild type (WT) and *au*, *hp* mutants grown on continuous light or in darkness was also determined (Fig. 3). The level of ACh in plants grown on light was higher than in the dark-grown seedlings. In the seedlings of wild type this difference was over 80 %. In case of the *hp* mutant it was

Fig. 2. The level and distribution of acetylcholine (nmole/g fresh weight) in different organs of 7-d-old etiolated WT tomato seedlings.
A - whole seedlings, B - cotyledons, C - hypocotyls, D - roots

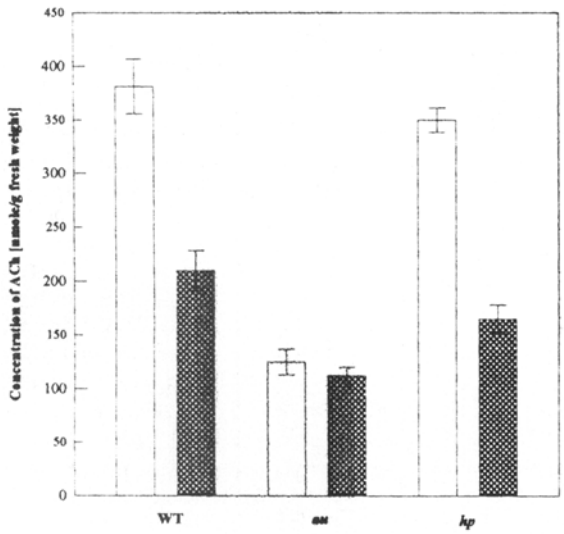


Fig. 3. The level of acetylcholine (nmole/g fresh weight) in WT, as well as in *au* and *hp* phytochrome tomato mutants cultivated for 7 days either in darkness (black bars) or under continuous white light (white bars).

even greater and exceeded 110 %. Only in *au* mutant no significant differences between the level of ACh in the light and dark-grown seedlings were observed (Fig. 3).

Fig. 4 shows the results of experiments conducted on *tri* and *fri* mutants. In wild type plants (WT-*tri* and WT-*fri*) grown on light the content of ACh was higher than in those grown in darkness (by 48 and 38 %, respectively). Comparing the level of ACh in phytochrome mutants a completely different situation was observed. In the tissues of *tri* no essential differences in the content of ACh in light- and dark-cultured seedlings were established (Fig. 4).

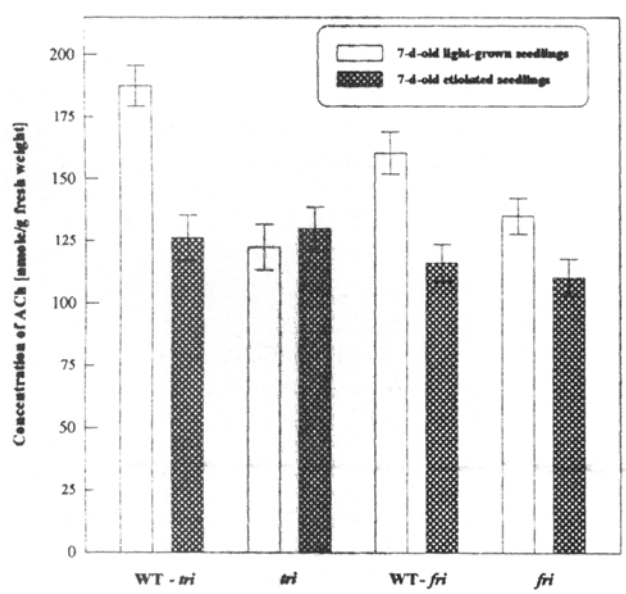


Fig. 4. The level of acetylcholine (nmole/g fresh weight) in *tri* and *fri* phytochrome tomato mutants (and their WT lines) cultivated for 7 days either in darkness or under continuous white light.

Contrary to *tri*, the seedlings of *fri* growing on light contained higher content of ACh than dark-grown seedling. It is also worth mentioning that the level of ACh in etiolated *tri* and *fri* seedlings was similar (130 and 110 nmole/g of fresh weight, respectively).

The effect of red light (R) and far-red light (FR) on the level of ACh in etiolated 7-day-old tomato seedlings of wild type, and in *au*, *hp* mutants were also investigated (Fig. 5). As controls etiolated, non-irradiated seedlings were used. In wild-type (WT) plants the content of ACh was considerably higher after 10-minute-long irradiation with R, however, 30 minutes after irradiation the concentration of this substance was lowered. The 20-min-long pulse of FR applied directly after R reversed that effect. Contrary to the seedlings of WT, in the *au* mutant no influence of R or FR on the level of ACh was found (Fig. 5). Changes in the level of ACh in the seedlings of *hp* mutant subjected to R and FR are illustrated in Fig. 5. In the mutant, contrary to WT plants, red light lowered the content of Ach by about 11 % . Its level was still being decreased during the next 30 minutes after irradiation *hp* seedlings with R. On the other hand, after sequential irradiation with red and far red light an insignificant increase in the content of ACh was observed. However, 30 minutes after these irradiations, a considerable drop of ACh level was noted (Fig. 5).

The changes in the level of ACh in etiolated tomato seedlings of wild type (WT-*tri*) and *tri* mutant after irradiation with R and FR are illustrated in Fig. 6. It has been stated that in WT-*tri* seedlings the content of ACh was increased as a result of irradiation with 10 minute long

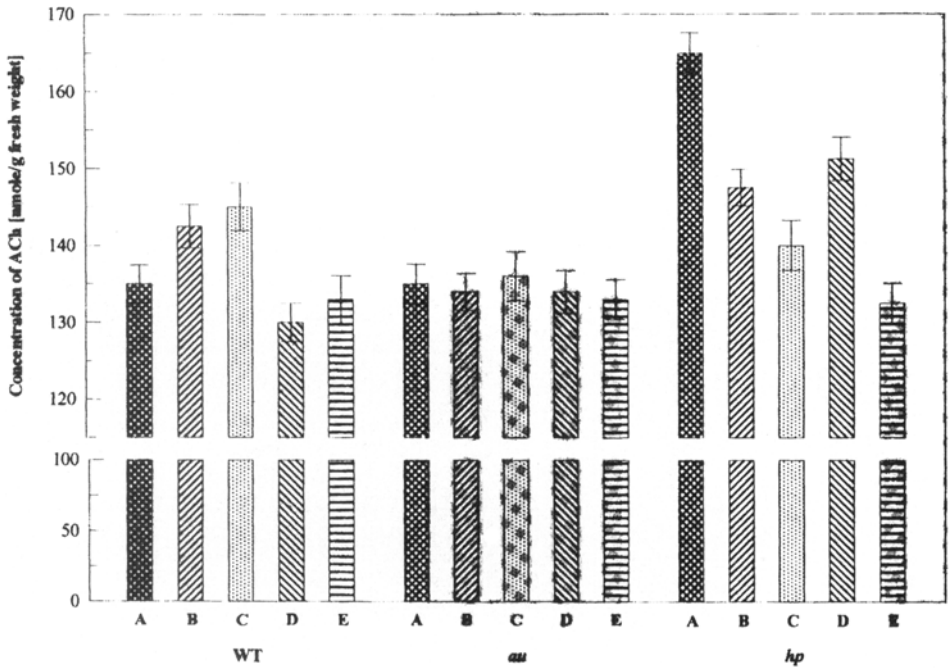


Fig. 5. The effect of red (R) and far red (FR) light on the level of acetylcholine (nmole/g fresh weight) in 7-d-old etiolated WT, *au* and *hp* phytochrome tomato mutants.
A - etiolated seedlings (control), B - red light irradiated seedlings (10 min), C - red light irradiated seedlings (10 min) - material collected 30 min after irradiation, D - red and far red light irradiated seedlings (10 min R + 20 min FR),
E - red and far red light irradiated seedlings (10 min R + 20 min FR) - material collected 30 min after irradiation

pulse of R, whereas 30 minutes after end of irradiation ACh content was slightly lowered. The effect of red light was reversed by 20-minute-long pulse of FR, applied directly after R (Fig. 6). In the seedlings of the mutant *tri* the red light caused a considerable increase in the level of ACh (by about 12 %). However, 30 minutes after the end of irradiation with R the concentration of ACh was decreased. Far red light reversed the effect of R action. After FR irradiation a considerable drop of the ACh content (by about 17 %) as compared with plants irradiated with red light was observed. During 30 minutes after the end of irradiation the level of ACh was again increased and reached the value observed in the tissues of control plants (Fig. 6).

Red light increased the content of ACh in the seedlings of WT-*fri*, and far red light reversed this effect. Directly after the irradiation of plants with FR the concentration of ACh was considerably lowered, and then, during next 30 minutes it returned to the level observed in the tissues of control plants (Fig. 7). Contrary to the plants of wild type (WT-*fri*), in the mutant *fri* 10-minute-long pulse of red light caused the decrease in the level of ACh. Thirty minutes after the irradiation

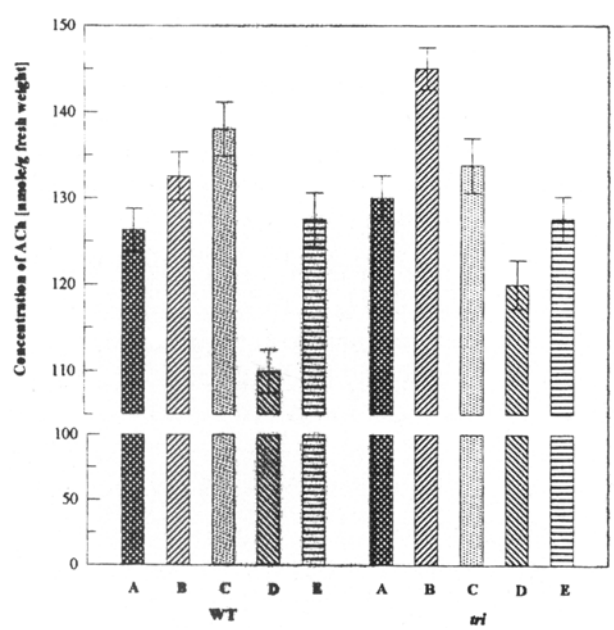


Fig. 6. The effect of red (R) and far red (FR) light on the level of acetylcholine (nmole/g fresh weight) in 7-d-old etiolated seedlings of *tri* (*cv.* GT) phytochrome tomato mutant.
A - etiolated seedlings (control), B - red light irradiated seedlings (10 min), C - red light irradiated seedlings (10 min) - material collected 30 min after irradiation, D - red and far red light irradiated seedlings (10 min R + 20 min FR), E - red and far red light irradiated seedlings (10 min R + 20 min FR) - material collected 30 min after irradiation

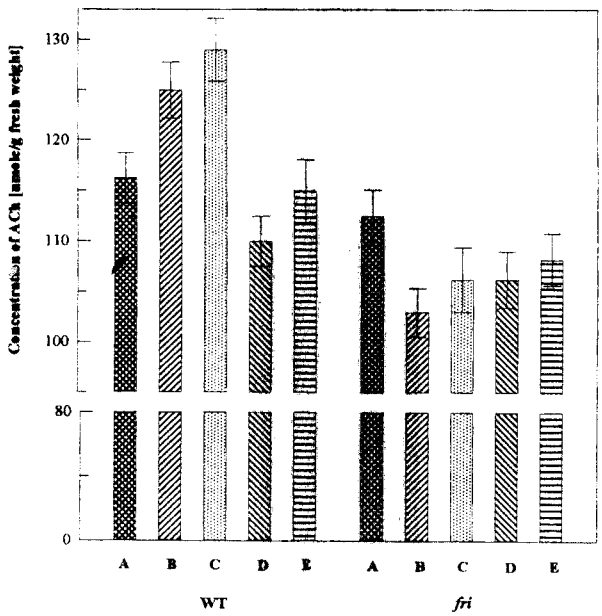


Fig. 7. The effect of red (R) and far red (FR) light on the level of acetylcholine (nmole/g fresh weight) in 7-d-old etiolated seedlings of *fri* (*cv.* MM) phytochrome tomato mutant. A - etiolated seedlings (control), B - red light irradiated seedlings (10 min), C - red light irradiated seedlings (10 min) - material collected 30 min after irradiation, D - red and far red light irradiated seedlings (10 min R + 20 min FR), E - red and far red light irradiated seedlings (10 min R + 20 min FR) - material collected 30 min after irradiation

with R the content of this substance slightly increased, however, it was still lower than in controls (Fig. 7). Also FR applied directly after R caused a slight increase in the level of ACh. But higher content of ACh was established in the material collected 30 minutes after the irradiation with both light wavelengths (Fig. 7).

Discussion

The content of ACh in tomato tissues as compared to other plant species is relatively high (Hartmann and Kiblinger 1974a, Miura and Shih 1984, Momonoki Y.S. and Momonoki T. 1991). Similarly as in other so far investigated plants (Jaffe 1970, Hartmann and Kiblinger 1974a, Miura and Shih 1984), in seven-day-old tomato seedlings the presence of ACh was discovered in all organs studied: cotyledons, hypocotyls and roots. Overground parts of the seedling contained more of ACh than underground ones, which is in agreement with the data obtained by Hartmann and Kilbinger (1974b). These authors showed that in *Pisum sativum* the content of ACh in shoots was equal to 8.2 nmole/g of fresh weight, whereas in roots it amounted to only 1,4 nmole/g of fresh weight. Momonoki Y.S. and Momonoki T. (1991) examining *Cucumis sativus* and *Vigna unguiculata* observed a higher level of ACh in stem than in leaves. In tomato seedlings the highest level of the investigated substance was noted in the youngest growing parts, that is in cotyledons (Fig. 2). Lin (1957) and Jaffe (1970) obtained similar results in *Artocarpus integra* and in *Phaseolus aureus*. In beans the highest concentration of ACh occurred in apical buds, in roots tops and in the young cotyledons. Cotyledons were also the richest in ACh organs in tomato. The lowest content of ACh was stated in roots. These results are in agreement with data obtained by Momonoki Y.S. and Momonoki T. (1991) for *Cucumis sativus* and *Vigna unguiculata*. However, in the case of *Raphanus sativus* the authors observed a inverse dependence - underground organs contained more ACh than the overground ones (Momonoki Y.S. and Momonoki T. 1991).

In tomato seedlings grown on continuous light the level of acetylcholine was higher than in etiolated ones. This regularity was observed in all the investigated cultivars of a wild type and *hp* and *tri* phytochrome mutants of tomato (Figs. 3 and 4). A similar effect of light on the content of ACh in plants was shown by Miura and Shih (1984) for beans seedlings, as well as by Tretyn A. and Tretyn M. (1990) for oat. Also Hartmann and Kilbinger (1974a,b) stated that light promotes the synthesis of ACh in *Pisum sativum* and in moss callus.

Both in *au* mutant, which is characterized by the lack of labile and stable forms of phytochrome (Kendrick *et al.* 1994, 1997; van Tuinen *et al.* 1995; Terry and Kendrick 1996), as well as in *tri* mutant, characterized by the lack of a stable form of phytochrome only (van Tuinen *et al.* 1996; Kerckhoffs 1996) no differences in the content of ACh between seedlings grown on light and in the dark were observed. These results suggest the participation of

phytochrome in the regulation of ACh level in tomato. This assumption was confirmed by the effect of R and FR irradiation on the content of ACh in the tissues of tomato phytochrome mutants. It was found that in the seedlings of wild type (WT) of all the varieties a 10-minute-long irradiation with R causes the increase in the level of ACh. Far red light applied directly after the pulse of R nullified this effect. In such case the content of ACh was considerably lowered, and after 30 minutes it returned to the level observed in the seedlings grown in darkness (see Figs. 5, 6, 7). These results are in agreement with earlier published data and confirm a generally accepted assumption that the level of ACh in plants is controlled by the phytochrome (Jaffe 1976, Hartmann and Gupta 1989, Tretyn and Kendrick 1991). In case of etiolated seedlings of several plant species it was observed that their irradiation with R or red light followed by FR is accompanied with the increase or the decrease of endogenous ACh level, respectively (Jaffe 1970, 1972, Hartmann and Kiblinger 1974a,b, Kopcewicz *et al.* 1977, Tretyn A. and Tretyn M. 1990). Thus, it was suggested that ACh may play role of a specific phytochrome transmitter or take part in phytochrome-controlled signal transduction chain.

It was stated in the presented work that in the seedlings of the *hp* mutant, contrary to WT plants, 10-minute-long pulse of R caused a considerable decrease of ACh level. Far-red light applied directly after red light slightly increased the content of this substance. Thirty minutes later, however, the content of ACh dropped (Fig. 5). It was shown that the *hp* mutant contains similar level of phytochrome to that present in WT seedlings (Kendrick *et al.* 1994), however, it has enhanced sensitivity to light treatment (Peters 1992). Therefore, it was postulated that mutation in *HP* gene leads to the perturbation in light-induced signal transduction (Kendrick *et al.* 1994). We believe that this is the reason of different sensitivity of *hp* and WT seedlings to red and far red light treatment.

The main aim of this work was to establish which of the physiological types of phytochrome is responsible for the regulation of ACh level in tomato. That is why, apart from *au* and *hp* also mutants of stable (*tri*) and labile (*fri*) phytochrome was used. It was shown, that *tri* is a mutant which does not have only one (PhyB1) out of three other (PhyB2, E, F) forms of stable phytochrome. However, it has also labile (PhyA) pool of the photoreceptor (van Tuinen *et al.* 1996, Kerckhoffs 1996). In *fri* mutant the labile phytochrome is absent, whereas it has all stable types of phytochrome (van Tuinen *et al.* 1995, Kerckhoffs 1996).

The results of our investigations showed that in *fri* mutant the level of ACh was higher on light as compared with etiolated seedlings, whereas in *tri* there were no differences (Fig. 4). On the base of the obtained results it may be supposed that a higher content of ACh in tissue of light-grown *fri* mutant is caused by the presence of a stable from of a phytochrome (PhyB), which probably controls the synthesis of this substance on continuous light. The lack of significant differences in the level of ACh in both light- and dark-grown *tri* seedlings suggests that synthesis of the substance is under PhyB1 control. It should be noted that the content of ACh in etiolated *fri* and *tri* seedlings was similar to its level in WT plants. It may suggest that the synthesis of ACh is not exclusively under phytochrome regulation. Because in green plants the synthesis of ACh probably takes place in chloroplasts (Roshchina and Mukhin 1985) the possibility that metabolism of acetylcholine is some-how linked with the light phase of photosynthesis (Roshchina 1987), could also can not be excluded.

The experiments in which the seedlings of *tri* and *fri* mutants were irradiated with red light and far red light supplied additional information on the role of a labile and stable form of phytochrome in the control of ACh turnover in tomato. In *tri* mutant the increase in ACh content was observed after the pulse of R, whereas FR reversed this effect (Fig. 6). A quite different situation was established in case of mutant *fri*, in which red light lowered the level of ACh and FR did not reverse this effect (Fig. 7).

The results of experiments conducted on different mutants of tomato suggest that both the forms of phytochrome take part in the control of ACh turnover. It is supposed that a labile form of photoreceptor is responsible for the fast synthesis of ACh in red light-irradiated etiolated seedlings. On the other hand, the synthesis of ACh in light-grown plants

seems to be controlled by a stabile form of phytochrome.

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